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Short communication

Flow cytometric characterization of Peyer's patch and cecal tonsil T lymphocytes in laying hens following challenge with *Salmonella enterica* serovar Enteritidis

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ABSTRACT

Two trials were conducted to determine T cell changes in Peyer's patches (PP) and cecal tonsils (CT) of specific-pathogen-free Single-Comb White Leghorn hens challenged with *Salmonella enterica* serovar Enteritidis (*SE*). Each week, crop lavage samples were obtained from 4 or 3 hens in Trials 1 and 2, respectively. These birds were then sacrificed and their intestinal tracts excised. The crop sample and contents of one cecum from each hen were cultured for the presence of *SE*. Cells were purified from proximal and distal PP along with both CT and then aliquots of cells were incubated with antibodies to CD4, CD8, and the three T cell receptors (TCR). The T subsets were identified via flow cytometric analysis. Crop and cecal samples were 100% culture positive for *SE* at week 1 post challenge and a percentage of samples remained positive throughout the study. Some differences in TCR subsets between or within tissues were observed at various times relative to *SE* challenge but over-all the subsets remained similar during the study. The predominant TCR was TCR2 (vβ1) followed by TCR3 (vβ2). Low numbers of TCR1 (γδ) cells were observed. CD4/CD8 ratios increased in the PP and CT tissues by week 1 post challenge and the ratio elevation persisted throughout the experiment. These results indicate that T cell populations are comparable between PP and CT and enteric *SE* infection can affect the cellular dynamics of these lymphoid tissues.

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1. Introduction

Salmonella remains a serious human food safety threat and much attention has been focused on identifying sources of the organism and then taking steps to eliminate the source from the food chain. Poultry and their products have been identified as an important *Salmonella* reservoir (Rabsch et al., 2001), and much time, research and resources have been devoted to developing methods to reduce the incidence of *Salmonella* in poultry flocks and therefore improve the microbial safety of products leaving the farm. An important tool used for reducing *Salmonella* in poultry is to increase the resistance of the bird to the organism through stimulation of the immune system. Vaccination (Lillehoj et al., 2000; Van Immerseel et al., 2005), cytokine/chemokine administration (Lowenthal et al., 2000), and immunomodulators (He et al., 2005; Lowry et al., 2005) have all been used to enhance resistance to infection and therefore reduce *Salmonella* flock presence. A more thorough understanding of the avian immune system should increase the potential for developing more effective methods to stimulate immunity and improve the chances for creating *Salmonella*-free flocks.

Salmonella infections can occur at many stages of a flock's life. Hatcheries have been shown to be an important site for the introduction (Bailey et al., 1994) and amplification (Cason et al., 1994) of a *Salmonella* infection.

Abbreviations: FITC, fluoresceine isothiocyanate; PBS, phosphate buffered saline; PP, Peyer's patch; R-PE, R-phycoerythrin; *S.*, *Salmonella*; TCR, T cell receptor; Cy5, Cyanine 5; XLT4, xylose lysine tergitol-4 agar; *SE*, *Salmonella enterica* serovar Enteritidis; BSL-2, biosafety level-2.

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In older birds, contaminated poultry house environments have been implicated in *Salmonella* outbreaks of newly place flocks (Yamane et al., 2000) and stress situations such as the onset of egg lay and induced molting increases the infection incidence (Riemann et al., 1998). Only a small percentage, approximately 10%, of hens in a flock are estimated to be infected with *Salmonella enterica* serovar Enteritidis (*SE*), leaving a large number of possible recipients for infection at later time periods (Ebel et al., 1992). Flock *SE* prevalence was shown to change over time (Wales et al., 2007) and production of contaminated eggs tended to be sporadic over the life of a flock (Humphrey et al., 1989b). Investigations into the development of immunity following *Salmonella* infection need to be conducted at a number of bird ages.

Peyer's patches (PP) are organized lymphoid tissues located at various sites along the small intestine (Makala et al., 2002). Generally characterized as a secondary lymphoid organ, the PP has been shown to be an important regulator and amplifier of mucosal immune responses to antigenic stimuli such as infection (Keren et al., 1978). Because the mammalian PP can be readily observed via the serosal surfaces of the bowel, a large volume of research has been conducted to define the role of this tissue in providing mammalian mucosal immune protection. This is in contrast to the avian PP which is much less grossly apparent in unfixed tissue. As a result, minimal information is available regarding PP in poultry especially with respect to its role in responding to and regulating the mucosal immune response against infection by intestinal pathogens. Implementation of a staining method which allows rapid identification of PP on the serosal surface of unfixed chicken ileum (Vaughn et al., 2006) will enable researchers to pursue a wide range of studies to delineate the role of the chicken PP in the elicitation of mucosal immune responses of the intestinal tract. Two PP were shown to predominate along the chicken lower alimentary tract, the proximal PP which was approximately 3–6 cm caudal to the Meckel's diverticulum and the distal PP which was 7–10 cm cranial to the ileocecal junction. The location of the two PP tissues in the upper and lower portions of the ileum, respectively, could potentially impact the degree of interaction with intestinal pathogens and these PP sites may exhibit different cellular dynamics during challenge.

Salmonellae are considered intracellular pathogens and, as a result, T cell immunity plays an important role in providing protective immunity against infection. T cell immunity is mediated and regulated by both CD4+ helper T cells (TH) and CD8+ cytotoxic T cells (TC). Within these two T cell populations, the cells may express one of three antigen receptors: TCRγδ (TCR1), TCRvβ1 (TCR2) and TCRvβ2 (TCR3). Different T cell subpopulations have been shown to be more prevalent in certain tissues and infection can change this cellular prevalence in tissues over time (Vervelde and Jeurissen, 1995; Yun et al., 2000). Previous work examined changes in gut immunological tissues following *Salmonella* infection in very young birds (Sasai et al., 2000; Berndt et al., 2007; Van Hemert et al., 2007) but little has been conducted in older, more mature individuals. As infections can occur in these older hens, with the increased risk of producing contaminated eggs, an understanding of immunity development in the intestinal tract of these individuals is an important step in establishing regimens to prevent flock *Salmonella* problems. The current investigation was undertaken to analyze, via flow cytometric analysis, the T cell repertoire of proximal and distal PP prior to and following *SE* challenge in hens in active egg lay and compare these populations with those found in cecal tonsils, another intestinal lymphoid tissue.

2. Materials and methods

2.1. Chickens

Single-Comb White Leghorn chickens, 34 and 41weeks of age in Trials 1 and 2, respectively, were obtained from the specific-pathogen-free flock maintained at the Southeast Poultry Research Laboratory (SEPRL), Athens, GA. The hens were transferred to individual adjacent laying cages in an environmentally controlled BSL-2 building at SEPRL and allowed to acclimate 7 days. The hens were fed layer ration *ad libitum* throughout the experiment duration. To assure that the hens were *Salmonella*-free, the individuals were screened for *Salmonella* prior to the commencement of experiments by enriching 1 g feces in 9 ml Rappaport-Vassiliadis (RV) enrichment medium (Oxoid Inc., Basingstoke, England), incubating overnight at 37 °C, and plating 100 μl of the broth onto XLT4 agar (Remel, Lenexa, Kansas). *Salmonella* was not detected. The studies were approved by and conducted under the guidelines of the SEPRL Institutional Animal Care and Use Committee.

2.2. Infection

Frozen stocks of nalidixic-acid-resistant *SE*, strain SE89-8312, were maintained at −20 °C. For each trial, 3 days prior to infection, the *SE* was thawed and cultured onto nutrient agar (Difco/Becton Dickinson Microbiology Systems, Sparks, Maryland) at 37 °C for 18–24 h. An individual colony of the *SE* was re-cultured onto nutrient agar and incubated at 37 °C for 18–24 h. A 10-ml tube of tryptic soy broth (Difco) was then inoculated with isolated colonies from the nutrient agar plate and incubated overnight at 37 °C. The *SE* broth culture was diluted 10^{-1} in sterile saline and each bird received a dose of 1 ml *per os* (6.9×10^7 and 5.3×10^7 *SE*, Trials 1 and 2, respectively). Crop lavage samples (Holt et al., 2002) were obtained from 4 hens/sampling day (Trial 1) and 3 hens/sampling day (Trial 2) on day 0 and weeks 1–4 by instilling 5 ml glycine buffer via tubing attached to a 10-ml syringe. The crop sample was aspirated back into the syringe and transferred to a 15-ml tube kept on ice. The birds were then euthanized via CO_2 inhalation and a portion of the left cecum, approximately 1 cm from the apex, was placed into a tared stomacher bag. The crop and cecum samples were transported back to the laboratory. One milliliter of each crop sample was added to 9 ml tetrathionate brilliant green (TBG) broth. A volume of TBG broth was added to each cecum tissue to dilute it 1:10 and then the tissues were emulsified via stomaching. The crop and cecum TBG samples were incubated for 24 h at 37 °C and then a loopful

of each sample was plated onto brilliant green agar containing 20 μg/ml nalidixic acid and novobiocin (BGNN). Following 24 h incubation at 37 °C, the plates were evaluated for the presence of *SE*.

2.3. Peyer's patch and cecal tonsil processing and staining

Intestines, approximately 5 cm cranial to the Meckel's diverticulum to 2.5 cm caudal to the ileocecal junction, were removed from the sacrificed hens alluded to above. Both CT were extirpated and combined into a tube containing chilled RPMI-1640 tissue culture media (Sigma Chemical Company, St. Louis, Missouri). The lumen of the ileum was thoroughly rinsed with three 20-ml distilled water flushes and the tissue was ligated at the most proximal (jejunum–ileum junction) using Carmalt forceps. The PP were visualized using a staining procedure previously described (Vaughn et al., 2006) that involved infusion of 10–20 ml diluted aqueous eosin-Y (Hema 3, Fisher Scientific, Suwanee, Georgia) into the gut lumen for 1 min, gently extruding the stain, and then briefly infusing the gut lumen with a diluted crystal violet stain (Sigma). Two PP could be observed on the ileum anti-mesenteric serosal surface, the proximal PP located 3–6 cm caudal to the Meckel's diverticulum and the distal PP located 7–10 cm cranial to the ileocecal junction. Both PP were extirpated and placed into separate tubes of chilled RPMI-1640 media. A six-well tissue culture plate (Costar, Corning Inc., Corning, New York) was used to collect cells from the PP and CT sites. The cells from each tissue were collected by mashing the tissue through a 70-μm nylon-strainer (Fisher Scientific) into 4 ml RPMI-1640. A further 2 ml media was used to rinse any remaining cells through the strainer. Cells were collected from the plate wells, gently mixed, centrifuged at 1500 RPM, and the supernatant aspirated. The cell pellet was then resuspended in RPMI-1640 and centrifuged at 1500 RPM an additional two times. Viable lymphocyte counts were made using the trypan blue exclusion method and the cells were diluted to a working concentration of 2×10^6 viable cells/ml, then 100 μl of cells were aliquoted and stained using cocktails containing: anti-chicken CD4 coupled to fluorescein isothiocyanate (FITC, 1.2 μg antibody/10^5 cells), anti-chicken CD8 coupled to Cyanine 5 (Cy5, 0.4 μg antibody/10^5 cells) and anti-chicken TCR1, 2 or 3 coupled to R-phycoerythrin (R-PE, 0.14 μg antibody/10^5 cells). All antibody reagents were purchased from Southern Biotech, Birmingham, Alabama. The cells were stained at 4 °C in the dark for 30 min and then washed 3×. At the completion of the staining and washing procedure, the cells were resuspended in 400 μl PBS/1% paraformaldehyde and allowed to fix overnight at 4 °C. Flow cytofluorometry was performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California). Five parameters, forward light scattering (FSC), sideward light scattering (SSC), FITC (FL1), PE (FL2), and Cy5 (FL4) were collected on the cells and evaluated using FlowJo analysis software (Tree Star Inc., Ashland, Oregon). Compensation for spectral overlap of each fluorochrome was performed using CompBead anti-mouse IgG κ beads (Becton Dickinson) incubated with the appropriate fluorochrome-labeled antibody reagent.

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Instat (GraphPad Software, Inc., San Diego, CA). Analysis of variance (ANOVA) with Tukey's multiple comparison test procedures were conducted for comparing T cell sub-populations between PP and CT tissues at each sample point, examining changes in T cell populations within same tissue types over time and comparing the CD4/CD8 ratios between tissues on each test day.

3. Results and discussion

Salmonellae are enteric organisms and preferentially colonize and invade the alimentary tract. This is reflected in the current study where both crop and ceca were 100 % culture positive for *SE* at 1-week post challenge in Trial 1 and then decreasing over the next 3 weeks. Similar results were observed in Trial 2 with both tissues being *SE* culture positive at 1 and 2 weeks post challenge and then decreasing thereafter.

Age affects the severity of infections in poultry by paratyphoid salmonellae such as *SE*. While extra-intestinal dissemination to internal organs can be readily observed, *Salmonella* infection in older birds will generally not present with readily observable clinical symptoms (Humphrey et al., 1989a,b). In contrast, chickens exposed to the organism in the first week post hatch exhibit increased morbidity and mortality (Gast and Holt, 1998), demonstrate poor immune responses to the bacteria (Holt et al., 1999), and, as a result, display difficulty clearing the organism over time. Significant changes in T cell subsets could be observed in CT (Sasai et al., 2000), the cecal mucosa (Berndt et al., 2007) or jejunum mucosa (Van Hemert et al., 2007) of chicks challenged with *Salmonella* at 1-day post hatch. Much less is known regarding the effects of *Salmonella* infection on lymphocyte populations in the alimentary tract of older birds. In both Trial 1 (Fig. 1) and Trial 2 (Fig. 2), the predominant TCR was TCR2 (αβ Vβ1) followed by TCR3 (αβ Vβ2). TCR1 (γδ) T cells were generally sparse in the PP and CT. CD4+ T cells were generally more prevalent than CD8, and CD4+TCR2+ was the most predominant cell type. For Trial 1, comparison of lymphocyte subsets between proximal PP vs. distal PP vs. CT tissues determined no significant differences at the various times post challenge. Similarly, little change was observed in lymphocyte subsets when the same tissue types were compared at various times post challenge, although CD8+TCR2+ lymphocytes of CT were significantly increased at week 4 post challenge ($P < 0.05$) after only a moderate increase seen at week 2 post challenge. In Trial 2, CD8+TCR2+ lymphocyte numbers were significantly higher in pre challenge proximal PP compared with CT ($P < 0.05$) but this was not observed 1 week later following *SE* challenge. Proximal PP CD4+TCR1+ numbers were significantly higher ($P < 0.05$) than distal PP or CT at 1-week post challenge. By week 2 post challenge, the

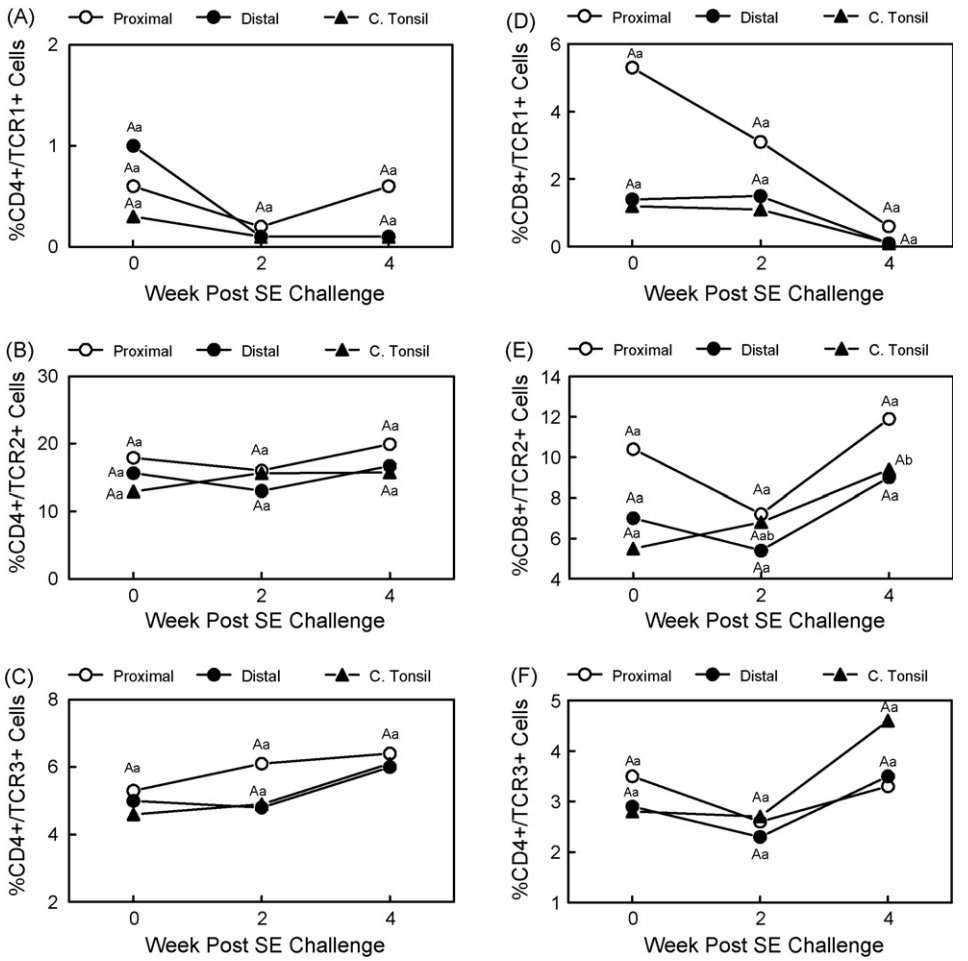

Fig. 1. Comparison of CD4+ (A–C) and CD8+ (D–F) T cell subpopulation changes in proximal PP (open circle), distal PP (filled circle), and cecal tonsils (C. Tonsil, filled triangle) at various times post *SE* challenge, Trial 1. Results represent percentages of each T cell subpopulation at different times in relation to *SE* infection ($n = 4$ birds/sample). [A]Letter change in superscripts within a line represents significantly different population counts between the tissues ($P < 0.05$). [a]Letter change in superscripts of a particular T cell subset within a tissue on different days in relation to *SE* infection represents significantly different count ($P < 0.05$).

proximal PP CD4+TCR1+ lymphocyte numbers decreased to levels similar to the distal PP and CT tissues, but this was significantly lower ($P < 0.05$) than that observed for the proximal PP tissue at the other time points. The less dramatic change in T cell populations observed following *SE* challenge in the current study as compared with that observed in previous reports (Sasai et al., 2000) may be a reflection of the age of the chickens, because the latter study used very young birds while the current study was conducted with adult hens.

The CD4/CD8 ratio can be used to provide a convenient standard for observing changes in cellular immune status during disease (Liu et al., 2002; Wang et al., 2003), nutritional stress (Lee and Woodward, 1996), and auto-immune problems (Kantrow et al., 1997). The CD4/CD8 ratio reflects changes in both T cell populations evaluated together at a particular time point, and therefore the ratio can be impacted more dramatically by smaller changes in each population. These changes might exert less observable impacts in the broader comparison, especially if the changes were spread throughout the CD4 or CD8 populations. Looking at changes within tissues following challenge, the CD4/CD8 ratio significantly increased in the proximal PP and the CT at 1-week post challenge ($P < 0.05$) and in all three tissues at week 2 (Fig. 3). The distal PP CD4/CD8 ratio remained significantly higher than its pre-challenge level at week 3 while proximal PP and CT decreased to levels no longer statistically significant. These results indicate that, while gross changes in the PP and CT lymphocyte subpopulations may be less obvious, *SE* infection does exert a dramatic effect on the CD4/CD8 dynamic in these tissues. Between tissues, the CD4/CD8 ratios were significantly ($P < 0.05$) higher in CT compared with proximal PP prior to infection and both the proximal and distal PP at 1-week post challenge ($P < 0.01$). Over the next few weeks, the ratio gradually decreased down to the levels observed for the distal PP but still significantly higher than the proximal PP ($P < 0.05$). The observed higher CD4/CD8 ratios in CT compared with PP may be a reflection of lymphoid tissue location. Located at the proximal end of each cecum, an organ possessing a large microbial flora, the CT are exposed to a constant influx of microbial antigens. This is reflected in

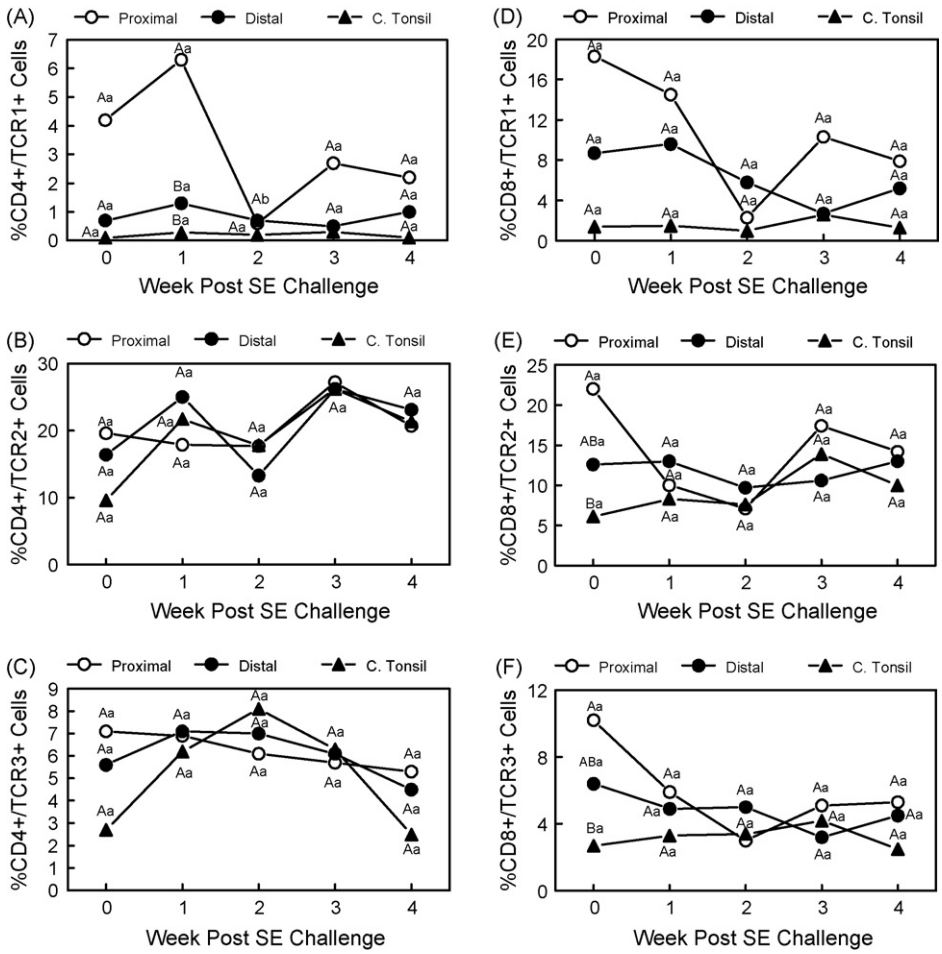

Fig. 2. Comparison of CD4+ (A–C) and CD8+ (D–F) T cell subpopulation changes in proximal PP (open circle), distal PP (filled circle), and cecal tonsils (C. Tonsil, filled triangle) at various times post *SE* challenge, Trial 2. Results represent percentages of each T cell subpopulation at different times in relation to *SE* infection ($n = 3$ birds/sample). [A]Letter change in superscripts within a line represents significantly different population counts between the tissues ($P < 0.05$). [a]Letter change in superscripts of a particular T cell subset within a tissue on different days in relation to *SE* infection represents significantly different count ($P < 0.05$).

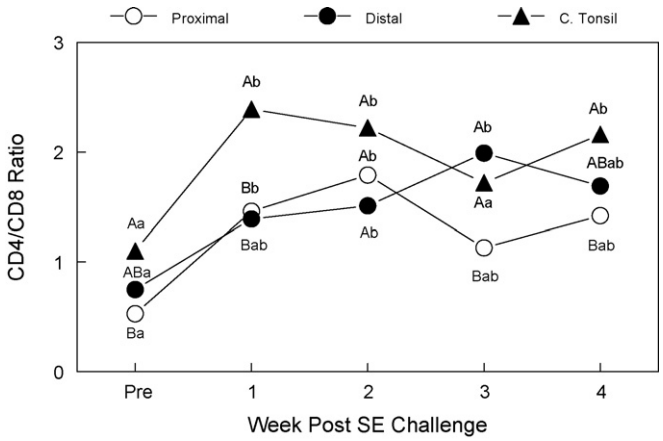

Fig. 3. CD4/CD8 lymphocyte ratios in proximal PP (open circle), distal PP (filled circle), and cecal tonsil (CT, filled triangle) at pre and weeks 1, 2, 3, 4 weeks post challenge ($n = 3$ birds/sampling day). [A]Letter change in superscripts within a sample day represents significantly different CD4/CD8 ratio between the tissues ($P < 0.05$). [a]Letter change in superscripts between sample days represents significantly different CD4/CD8 ratio within a tissue ($P < 0.05$).

studies by Hedge et al. (1982) who showed that microbial flora stimulation was essential for full lymphoid development in that tissue.

Earlier immunohistochemical studies showed a predominance of TCR2 cells in PP (Bucy et al., 1988) and CT (Cihak et al., 1991). In the present study, the primary TCR type was also TCR2 (αβ Vβ1) followed by TCR3 (αβ Vβ2), reinforcing those earlier studies. Additionally, in the current study, CD4+TCR2+ T cells predominated in PP, similar to that reported by Bucy et al. (1988). The presence of TCR1 (γδ) T cells, specifically CD4+ TCR1+ cells, were very low in PP and CT. This finding within PP and CT lymphoid tissues is in contrast with intestinal epithelium and lamina propria which possess higher levels of TCR1+ (γδ) lymphocytes (Bucy et al., 1988; Lillehoj, 1993).

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